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Enhancement of Biofilm Formation by Subinhibitory Concentrations of Macrolides in *icaADBC*-Positive and -Negative Clinical Isolates of *Staphylococcus epidermidis*[▽]

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Biofilm formation in *Staphylococcus epidermidis* is mediated by *icaADBC*-dependent and -independent pathways. Subinhibitory concentrations of erythromycin, azithromycin, and clarithromycin enhanced, in a dose-dependent manner, the level of biofilm formation by 20% (21/105 isolates) by macrolide-resistant *ica*-positive and -negative isolates tested *in vitro*. The presence of *ica*, however, apparently produced an enhanced effect on biofilm formation. The levels of expression of the biofilm-related genes *icaA*, *atlE*, *fruA*, *pyrR*, *sarA*, and *sigB* were increased in response to erythromycin. The results likely underscore the potential clinical relevance of macrolide-induced biofilm growth.

Staphylococcus epidermidis, a key nosocomial pathogen, is frequently associated with the use of indwelling medical devices and forms biofilms (9). The latter is mediated by *icaADBC*-dependent and -independent pathways (8, 17). The *ica* operon encodes enzymes that are involved in the production of polysaccharide intercellular adhesion (PIA), which mediates the intercellular adherence of bacteria and the accumulation of multilayer biofilms (8). Nevertheless, *ica* is not ubiquitously distributed in *S. epidermidis* (5, 8, 21), and *ica*-negative isolates also produce biofilm (19). Furthermore, the impact of exogenous substances, such as antibiotics, on bacterial biofilm formation has also drawn much attention (3, 6, 7, 11, 12, 23), although the numbers of studies performed with *S. epidermidis* are still limited. Macrolides, which include erythromycin (ERY), clarithromycin (CLR), and azithromycin (AZM), are among the most frequently used antibiotics in clinical settings. A subinhibitory concentration of ERY induced *ica* expression (20), suggesting a possible role of macrolides in biofilm formation, but the eradication of the slime-like glycocalyx matrix and, thus, the eradication of antibiofilm activity by a subinhibitory concentration of CLR in a CLR-resistant isolate of *S. epidermidis* (whose *ica* status was unknown) were also reported (24). The study described here was thus carried out to investigate the role of macrolides in biofilm formation and its relation to the *ica* status of *S. epidermidis*. We show that a large number of clinical isolates of *S. epidermidis* had significantly enhanced levels of biofilm formation after treatment with macrolides and that biofilm formation was independent of the *ica* status.

Of 121 *S. epidermidis* strains isolated from our hospital during the period from August 2007 to December 2008, 105 isolates (87%) were resistant to ERY (MIC > 8 μg/ml), with most of the isolates being highly resistant to ERY, CLR, and AZM (MICs ≥ 128 μg/ml), although they were susceptible to vancomycin (VAN; MICs = 1 to 2 μg/ml). By using a microtiter plate assay for biofilm formation (1) with cells grown in tryptic soy broth medium, ERY at 1/4× MIC (i.e., over the range of 4 to 32 μg/ml) was found to significantly enhance the level of biofilm formation in 20% (21/105) of the ERY-resistant isolates (Tables 1 and 2). Two other macrolides, AZM and CLR, also caused a similar enhancement of biofilm formation (Table 2). VAN at 1/4× MIC affected the biofilm formation of only one isolate, in which biofilm formation was significantly inhibited. Dicloxacillin and tetracycline at 1/2× to 1/4× MICs also had no detectable effect or caused only limited inhibitory effects on biofilm formation. The biofilm growth of four ERY-susceptible isolates (ERY MICs = 0.06 to 1 μg/ml) tested was not affected by a subinhibitory concentration of ERY (data not shown).

PCR was performed to determine the *icaA* status of the isolates, tested by using the genomic DNA and the primers listed in Table 3 (including *gyrB* as a control), followed by analysis of the PCR products by agarose gel electrophoresis and/or DNA sequencing. The *icaA* gene was present in ca. 61% of the 105 ERY-resistant isolates and 52% of the 21 isolates in which biofilm production was enhanced by ERY (Tables 1 and 2). No correlation between macrolide-enhanced biofilm formation and the *icaA* status was established by a chi-square test (Tables 1 and 2).

Two isolates, *icaA*-positive isolate SW029 and *icaA*-negative isolate SW036, were selected for determination of the dose-related effect of the macrolides on biofilm growth. Both strains showed high levels of resistance to macrolides: ERY and AZM MICs were >128 μg/ml for both isolates, and CLR MICs were >128 and 16 μg/ml for SW029 and SW036, respectively. The representative biofilm growth of the two isolates in the presence of ERY and VAN is shown in Fig. 1. When a series of subinhibitory concentrations of macrolides (1/4×, 1/16×, and 1/64 MICs) were tested, a dose-dependent effect was clearly observed for SW036 (Fig. 2). Lower concentrations of ERY (1/256× and 1/1,024× MIC) were also tested with SW029. The

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TABLE 1. Effect of ERY on biofilm growth of clinical isolates of *S. epidermidis*

Strains (no. tested)	ERY MIC (μg/ml)	% with *icaA*	Relative biofilm formation[a]
Total isolates (105)	≥16	61 (64/105)[b]	1.32 ± 0.81[c]
Isolates with ERY-induced biofilm (21)	≥32	52 (11/21)	2.51 ± 1.53
Isolates lacking ERY-induced biofilm (84)	≥16	63 (53/84)	1.02 ± 0.13

[a] Biofilm formation was determined by a microtiter plate assay by measurement of the OD_{590} values. Relative biofilm formation is expressed as the ratio (mean ± standard deviation) of the OD_{590} values for 1/4× MIC ERY-treated to ERY-untreated isolates.

[b] Values in parentheses are the number of isolates with *icaA*/total number of isolates tested.

[c] A statistically significant difference was observed between the ERY-untreated and ERY-treated groups ($P < 0.05$ by paired-samples *t* test), suggesting a trend of macrolide-enhanced biofilm formation.

biofilm growth induced by ERY at 1/256× MIC was only half of that observed (i.e., from the values of the optical density at 590 nm [OD_{590}]) with ERY at 1/64× MIC, while ERY at 1/1,024× MIC did not induce biofilm formation. These results also demonstrate the dose-dependent biofilm induction for SW029. Together, the data prompt us to hypothesize that the presence of *ica* apparently produces enhanced biofilm formation, which is speculated to result from the potential synergistic interplay between the *ica* gene-dependent pathway and induction by macrolides. Nevertheless, the latter hypothesis remains to be tested, e.g., with isogenic strains. We also compared the biofilm formation of these isolates after the initial treatment with ERY for 6 h and the subsequent removal of ERY through repeated washing of the cells. Cells pretreated with ERY, regardless of their *ica* status, showed elevated levels of biofilm production at 18 h after ERY treatment (ca. 173% and 100% increases for SW029 and SW036, respectively), suggesting that the action of macrolides in biofilm enhancement occurs within 6 h and persists as a postantibiotic effect for a longer period.

The process of biofilm formation was further assessed with isolate SW029 by obtaining microscopic observations with a confocal laser scanning microscope (Leica, Germany) and a scanning electron microscope (S3400; Hitachi, Japan). For confocal laser scanning microscopy (CLSM), biofilm cells grown on coverslips (1 cm by 1 cm) were fixed with 2.5% glutaraldehyde and washed with phosphate-buffered saline, and concanavalin A conjugated to fluorescein isothiocyanate green (Sigma-Aldrich) was added at a concentration of 50 μg/ml, followed by staining with pyridoxylidense isoleucine (Sigma-Aldrich) at a concentration of 5 μg/ml. Adherent bacterial cells were observed by CLSM at 1, 6, and 12 h after incubation with ERY (i.e., each group included five slides, and

TABLE 2. Macrolide-enhanced biofilm formation by clinical isolates of *S. epidermidis*

Strain	Source	*icaA* presence	% biofilm formation enhanced by[a]:		
			ERY	AZM	CLR
ATCC 35984[b]	ATCC	+	7.0	2.5	−3.9
SW027	Urine	+	60.4	49.1	62.3
SW029	Blood	+	159.6	157.9	146.4
SW032	Eye secretion	+	78.5	71.8	71.8
SW045	Wounds	+	47.2	33.4	32.2
SW058	Blood	+	37.9	37.6	54.9
SW061	Catheter	+	50.8	58.4	46.3
SW067	Blood	+	150.3	128.8	140.2
SW085	Wounds	+	297.8	298.3	275.8
SW098	Wounds	+	93.9	104.0	112.8
SW104	Bile	+	63.2	48.1	51.5
SW126	Blood	+	56.1	56.4	50.3
Subtotal for *ica*-positive isolates ($n = 11$)[c]			99.6 ± 77.2	94.9 ± 78.3	95.0 ± 71.5
SW015	Wounds	−	87.8	57.3	78.6
SW021	Blood	−	34.7	39.8	54.6
SW036	Eye secretion	−	284.3	289.2	292
SW070	Blood	−	172.2	92.3	155.6
SW071	Wounds	−	230.4	277.8	147.2
SW082	Urine	−	58.7	59.8	62.8
SW088	Blood	−	163.1	117.2	201.5
SW099	Eye secretion	−	55.7	47.0	38.7
SW115	Blood	−	166.8	184.3	164.0
SW120	Neurolymph	−	69.4	75.8	50.6
Subtotal for *ica*-negative isolates ($n = 10$)[c]			132.3 ± 83.8	124.1 ± 94.0	124.6 ± 82.1

[a] The biofilm formation enhancement is expressed as [(biofilm formation in the presence of a macrolide − biofilm formation in the absence of a macrolide)/biofilm formation in the absence of a macrolide] × 100. Biofilm formation was measured by a microtiter plate assay by the determination of OD_{590} values. The data shown are the averages for quadruplicate samples.

[b] This reference strain did not show detectable macrolide-enhanced biofilm formation.

[c] No statistically significant difference ($P > 0.05$ by independent-samples *t* test) was observed between the two sets of subtotal values of the level of biofilm formation enhancement for *icaA*-positive and *icaA*-negative isolates.

TABLE 3. Primers used for PCR and RT-PCR in this study

Gene	Primer sequence (5′–3′)	Amplicon size (bp)
atlE	Forward: AACGAAGCAAGTAGCACC Reverse: ACACCACGATTAGCAGAC	108
fruA	Forward: GTGCAGGTTGCATGTCTA Reverse: AAGTGACCCTGTATCGTTTA	179
gyrB	Forward: AAGGGTATTATGGCTTCACG Reverse: TTTCACTTTCTTCAGGGTTC	139
	Forward: TTCGCATACGTTAATAAGTTGG Reverse: TGACGAGGCATTAGCAGGT	675
icaA	Forward: AGTTTCAGGCACTAACATCC Reverse: CGCAGTTACAGGTAATCCAC	295
	Forward: GAGGGAATCAAACAAGCA Reverse: AGGCACTAACATCCAGCA	405
pyrR	Forward: TATAGGGAGTTCACGATGA Reverse: CGGACGTACAGTAAGAGC	106
sarA	Forward: ATTTGCTTCTGTGATACGGT Reverse: TGAACACGATGAAAGAACTG	103
sigB	Forward: TACTCTAAGGGACAATCACATC Reverse: GGTACTAAGAAGGCTTCAAACT	119

the fields of view of each slide were positioned to the four corners and the center of each slide, respectively; the averages of the cell counts per field of view were then determined and statistical analysis was performed), while those cells observed at 24 and 48 h were assessed by scanning electron microscopy (SEM) (Fig. 3). There was no significant difference in the level of polysaccharide secretion (stained green) at 1 or 6 h when ERY was absent or present. However, ERY-treated cells yielded much more polysaccharide (i.e., they were thicker) at 12 h. The observations made by SEM also revealed that the matrix out of the cells increased for ERY-treated cells at both 24 and 48 h and also that ERY-treated cells displayed larger amounts of multilayer biofilms at 48 h (Fig. 3). While no

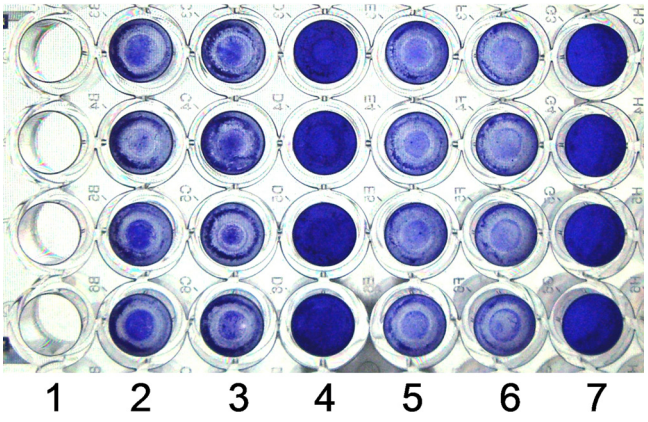

FIG. 1. Biofilm formation by *icaA*-positive isolate SW029 (columns 2 to 4) and *icaA*-negative isolate SW036 (columns 5 to 7) determined by a microtiter plate assay. Columns: 1, blank; 2 and 5, no antibiotics; 3 and 6, VAN at 1/4× MIC (0.25 and 0.5 μg/ml, respectively); 4 and 7, ERY at 1/4× MIC (32 μg/ml). The photo was taken from the back of the plate.

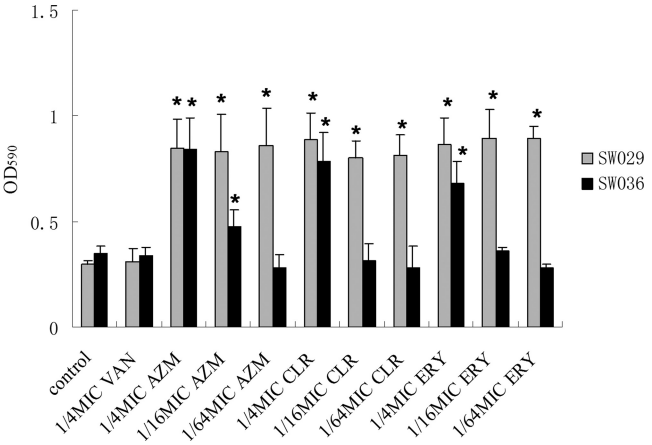

FIG. 2. Dose-dependent effects of macrolides at subinhibitory concentrations on biofilm formation of *icaA*-positive isolate SW029 and *icaA*-negative isolate SW036 determined by a microtiter plate assay. *, compared with the results for the relevant controls, $P < 0.05$.

significant difference was observed by counting the adherent bacteria in the presence or the absence of ERY at 1 h by CLSM (data not shown), CLSM showed that the level of biofilm formation increased significantly from 6 to 12 h. These data suggest that at subinhibitory concentrations, macrolides may not affect the initial adhesion of cells to the coverslips, despite their ability to induce biofilm formation.

Multiple genes have been known to be involved in biofilm formation in bacteria; and these include, for example, *icaADBC* (8, 20), mentioned above, as well as *atlE* (which encodes a major autolysin) (10), *fruA* (which encodes fructose-specific permease) (2, 16), *pyrR* (which encodes a pyrimidine regulatory protein) (2, 14), *sarA* (which encodes a global regulator) (4, 17, 22), and *sigB* (which encodes a sigma factor) (4, 18). To assess whether the expression of these genes was affected by macrolides, real-time reverse transcriptase (RT)-PCR was carried out with RNA templates prepared from the planktonic cells of isolates SW029 and SW036 in response to ERY at 8 μg/ml for 6 h or 24 h (*gyrB* was used as the reference gene). First-strand cDNA was synthesized, and PCR was preformed with an RT-PCR kit (Promega, Shanghai, China) and by SYBR green staining with a model 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The level of expression of each target gene was normalized to that of *gyrB*, and the relative gene expression was calculated from the ratio of their level of expression in ERY-treated cells to their level of expression in ERY-untreated cells. The results generally revealed elevated levels of expression of these genes in ERY-treated cells of both isolate SW029 and isolate SW036 (Table 4). In SW029, the *atlE* and *sarA* genes showed the highest levels of expression at 6 h and 24 h, respectively. Despite the increased level of gene expression after ERY treatment, *atlE* and *sigB* were relatively downexpressed and *icaA* and *sarA* were upexpressed from 6 h to 24 h in SW029 (Table 4). Such a comparison of gene expression likely provides insight into the possible mechanism(s) that leads to macrolide-enhanced biofilm growth. The elevated level of *icaA* expression over time from 6 to 24 h is consistent with the findings of other studies, which have demonstrated a role of the *ica* genes in biofilm

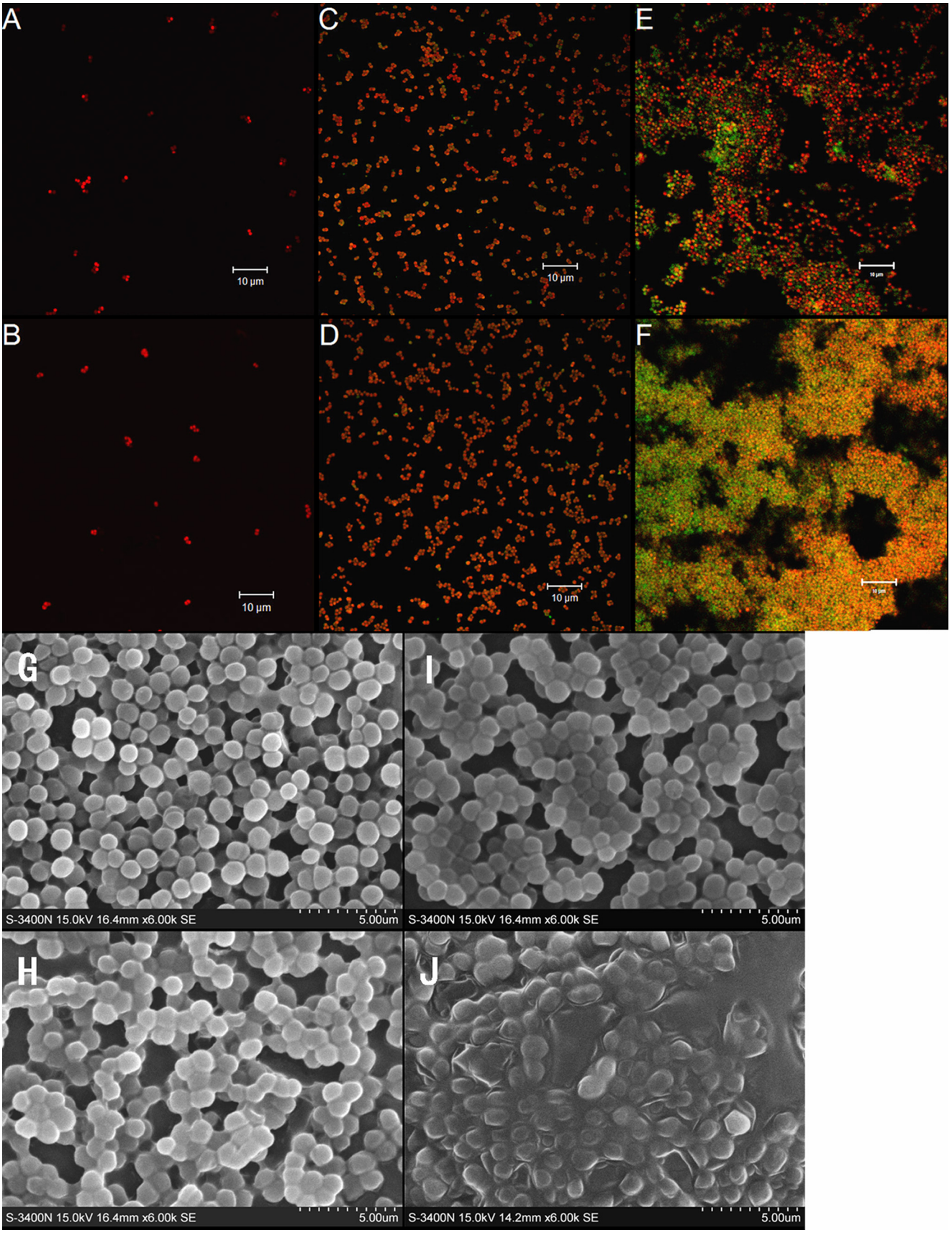

FIG. 3. Microscopic observation of biofilm formation of *ica*-positive isolate SW029 in the absence (A, C, E, G, and I) or presence (B, D, F, H, and J) of ERY at 1/4× MIC. (A to F) At time points of 1 h (A and B), 6 h (C and D), and 12 h (E and F), bacterial cells were observed by CLSM. The polysaccharides are stained green, and the cells without polysaccharides are stained red. Bars, 10 μm. (G to J) At 24 h (G and H) and 48 h (I and J), cells/biofilms were observed by SEM. Bars, 5 μm.

TABLE 4. Relative expression of several biofilm-related genes in *S. epidermidis* in response to ERY (6 h or 24 h posttreatment) assessed by real-time RT-PCR

Gene	Relative gene expression[a]		
	SW029 (*ica* positive)		SW036 (*ica* negative), 6 h
	6 h	24 h	
icaA	1.2	2.8	Not applicable
atlE	6.2	2.4	1.8
fruA	4.5	Not determined	2.4
pyrR	3.7	Not determined	2.4
sarA	1.7	6.9	1.7
sigB	2.0	0.6	1.4

[a] The relative gene expression is the ratio of the level of gene expression (measured by real-time RT-PCR) of cells treated with ERY to the level of gene expression of untreated cells.

formation (8, 17). The differential levels of expression of *atlE* and *sarA* also agree with the earlier observation that SarA affects the staphylococcal accessory gene regulator (Agr) system, a multicomponent signal transduction system that can influence biofilm growth and various virulence factors and that is affected by the cell growth environment (8, 17). The upregulation of these six genes was also observed in our preliminary DNA microarray assay, carried out with isolate SW029 using gene chips designed on the basis of the genome of *S. epidermidis* ATCC 35984 (strain RP62a) (Roche NimbleGen, Inc., Madison, WI) (data not shown). In particular, the increased level of expression of *atlE*, which encodes a major autolysin for primary staphylococcal adherence (10, 13), at 6 h likely not only suggests the action time of macrolides in inducing biofilm growth but also further supports the involvement of an *ica*-independent pathway in biofilm formation (10, 17).

Despite the observations presented above, the exact mechanism(s) responsible for macrolide-enhanced biofilm formation in *S. epidermidis* remains to be characterized. In *Pseudomonas aeruginosa*, an aminoglycoside response regulator named *arr* has been identified for aminoglycoside-enhanced biofilm formation (11). A recent report showed that furanones at subinhibitory concentrations enhanced the biofilm formation of *S. epidermidis* via the increased production of PIA (15). Finally, the macrolide-induced biofilm formation demonstrated in this study may have potential clinical implications, particularly for medical device-related bacterial biofilm infections, since the levels of macrolides at which *S. epidermidis* biofilm formation is enhanced are readily achievable in clinical settings. Further studies of macrolide-induced biofilm growth in *S. epidermidis* are warranted.

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